HHS Public Access

Author manuscript

Arch Virol. Author manuscript; available in PMC 2020 March 20.

Published in final edited form as:

Arch Virol. 2018 December ; 163(12): 3351–3356. doi:10.1007/s00705-018-4003-7.

Flanders hapavirus in western North America

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Abstract

Flanders virus (FLAV; family *Rhabdoviridae*) is a mosquito-borne hapavirus with no known pathology that is frequently isolated during arbovirus surveillance programs. Here, we document the presence of FLAV in *Culex tarsalis* mosquitoes and a Canada goose (*Branta canadensis*) collected in western North America, outside of the currently recognized range of FLAV. Until now, FLAV-like viruses detected in the western United States were assumed to be Hart Park virus (HPV, family *Rhabdoviridae*), a closely related congener. A re-examination of archived viral isolates revealed that FLAV was circulating in California as early as 1963. FLAV also was isolated in Nebraska, Colorado, South Dakota, North Dakota, and Saskatchewan, Canada. Phylogenetic analysis of the U1 pseudogene for 117 taxa and eight nuclear genes for 15 taxa demonstrated no distinct clustering between western FLAV isolates. Assuming the range of FLAV has been expanding west, these results indicate that FLAV likely spread west following multiple invasion events. However, it remains to be determined if the detection of FLAV in western North America is due to expansion or is a result of enhanced arbovirus surveillance or diagnostic techniques. Currently, the impact of FLAV infection remains unknown.

Flanders virus (FLAV) is a mosquito-borne hapavirus (genus *Hapavirus*; family *Rhabdoviridae*) that was first isolated in Flanders, New York, from *Culiseta melanura* mosquitoes and the spleen of an ovenbird (*Seiurus aurocapilla*) in 1961 [1]. It is commonly found in ornithophilic mosquitoes and avian hosts, such as house sparrows (*Passer domesticus*) and red-winged blackbirds (*Agelaius phoeniceus*) [1, 2]. Until 2016, the taxonomic classification of FLAV was unresolved, and it was listed as a member of the Hart Park serologic group, named after Hart Park virus (HPV; family *Rhabdoviridae*), a closely

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related congener that was first isolated from *Culex tarsalis* mosquitoes in Hart Park, California, in 1955 [3]. FLAV and HPV are now classified in the genus *Hapavirus* along with 13 other viruses. Historical isolations of FLAV and HPV have suggested that these viruses existed in allopatric populations where FLAV was restricted to eastern North America and HPV was restricted to western North America [2, 4–6]. FLAV and HPV are not associated with any apparent pathology, but the motivation to understand how nonpathogenic parasite communities influence disease dynamics continues to grow [7, 8].

In 2011, four FLAV-positive pools of *Cx. tarsalis* mosquitoes were identified from collections made on Aug. 2, 3 and 17 near cattail wetlands in eastern Larimer County, Colorado. Additional isolates were made from *Cx. tarsalis* from nearby locations in Colorado in 2013 [9]. Virus detected by Vero cell plaque assay screened negative for West Nile virus (WNV) based on WNV-specific RT-PCR and the VecTest Antigen Detection Assay® [10]. Further RT-PCR tests using group-reactive primers were negative for flaviviruses and alphaviruses but positive for Hart Park antigenic complex viruses. Sequenced amplicons aligned closely (97% identity) with the sequence of FLAV strain M11760 in the NCBI GenBank database (Accession no. JX431885.1). With no definitive record of FLAV in states west of Missouri and Texas, the isolation of FLAV in Colorado outside of its previously known range either indicated viral expansion or is a consequence of enhanced arbovirus surveillance. Here, we update the status of FLAV in western North America and use phylogenetic methods to understand relationships between western and eastern FLAV isolates.

Mosquito-derived FLAV and HPV isolates archived in − 80 °C freezers between 1963 and 2012 were opportunistically collected and sequenced for phylogenetic analysis (Table 1, Figures 1 and 2). Next-generation sequencing methods were applied to total RNA extracts. Custom RNA-Seq libraries were constructed as described previously. These RNA molecules were sequenced on an Illumina HiSeq platform (100 bp, PE), and viral genome sequences were assembled *de novo* and identified using the Virominer computational pipeline [11, 12].

Bayesian and maximum-likelihood (ML) methods were used to elucidate the evolutionary relationship between newly sequenced FLAV isolates and FLAV isolates registered in the NCBI GenBank database [4]. The nucleotide coding sequence for the U1 pseudogene was analyzed for 117 FLAV isolates. Additionally, a full genome analysis was completed for 15 FLAV isolates using the nucleotide coding sequence for structural proteins (N,P,M,G,L) and pseudogenes (U1,U2,U3) [4]. Structural proteins and pseudogene sequences were concatenated into a single file and aligned using the MAFFT L-INS-i algorithm [13]. Protein codon positions were visually inspected and aligned using Mesquite (Mesquite Project, version 3.04, released August 2015, accessed March 2016). Partition Finder was used to estimate the most appropriate model of genetic evolution for each structural gene partition, using the Akaike information criterion [14]. Bayesian analysis carried out using the program MrBayes employed a Markov chain Monte Carlo (MCMC) analysis with 50,000,000 generations, a print frequency of 1000, a sample frequency of 1000, and a 10% burn-in [15]. ML tests to confirm tree topology were performed on the CIPRES web server using the program RAxML with 1000 bootstrap replicates [16].

We characterized and sequenced a set of unpublished FLAV strains. These strains originated from the western USA and Canada, all from *Cx. tarsalis*, except for one isolate (NE00–23) from blood of an apparently healthy Canada goose (*Branta canadensis*; Table 1). Additionally, a nearly complete FLAV genome sequence (CA12–1161) was assembled *de novo* from a WNV-positive *Cx. tarsalis* pool (n = 50). The genome of a hapavirus isolate from Utah collected in 1983 could not be sequenced using our methods.

Based on phylogenetic analysis of the U1 pseudogene, newly sequenced western FLAV isolates appeared to originate from FLAV lineage A as defined by Allison et al. [4], with no observable phylogenetic clustering (Fig. 1). Within lineage A, 83V6207 (North Dakota) and M820–67 (Saskatchewan) appeared to form a distinct clade with 0.99 posterior probability support. Additionally, NE00–023 (Nebraska), CO11-P247 (Colorado) and CO11-P336 (Colorado) appeared to form a distinct clade with isolates collected in Georgia and Texas with 0.63 posterior probability support. CO1987 formed a distinct clade with DKB133–03, which was collected in Georgia in 2003 with a posterior probability support value of 1. CA63V-162 (California) and CT-460 (South Dakota) did not group with any other FLAV U1 sequence, and each formed its own phylogenetic clade with a posterior probability support value of 1 (Fig. 1).

Based on full-genome phylogenetic analysis, NE00–023 (Nebraska), CO11-P247 (Colorado) and CO11-P336 (Colorado) appeared to form a distinct clade in relation to all other western isolates, with a posterior probability of 0.98 (Fig. 2). CA63V-162 (California), CT-460 (South Dakota), BE-AN-781455 (Brazil), M11750 (USA) and 61_7484 (USA) formed a distinct clade with a posterior probability of 0.72.

Our study documents the extension of the known distribution of FLAV throughout western North America (Fig. 3) ([1, 3, 17–29]; GenBank accession numbers KJ958896.1 and KF028673.1). This extension may reflect improved molecular diagnostics and increased arbovirus surveillance in certain regions. The isolation of FLAV from a pool of *Cx. tarsalis* collected in California in 1963 indicated that viral transmission occurred in western North America at least 55 years ago and was not geographically restricted to the eastern USA as previously thought [4–6]. HPV was commonly isolated by the California Department of Health Services until the program shifted testing methods from intracerebral suckling mouse inoculation to Vero cell culture in the year 2000 and then to even more specific molecular methods [5, 30–32]. Although the available methods can distinguish between HPV and FLAV, isolations of FLAV in western North America may be misdiagnosed as HPV due to identification by broadly reacting antibodies and minimal interest in further characterization because of the apparently benign role of these viruses.

Phylogenetic analysis suggested that recent isolates from the Great Plains (CO11-P336, CO11-P247 and NE00–023) belong to a different strain than previous Great Plains isolates from Colorado, South Dakota, North Dakota, Canada or isolates from California (Fig. 2). However, whether FLAV was introduced to western North America on multiple occasions within the past 50 years from the eastern USA or if FLAV has remained endemic to the west remains undetermined. Even if FLAV has persisted in the western USA over the past 50 years, the absence of phylogenetic clustering suggests that there may be genetic exchange

between eastern and western North America (Fig. 1), perhaps due to the movement of mosquitoes and/or birds between eastern and western regions of the USA. Cross-infection between overwintering migratory birds in the tropics could also lead to the dispersal of FLAV between western and eastern populations. Metadata from sequences available in the GenBank databank (accession no. KJ958896) have documented the isolation of FLAV from Brazilian birds. Additionally, FLAV has been documented in parts of Mexico [23].

The association of FLAV with both ornithophilic mosquitoes and birds supports earlier suggestions that FLAV is an arbovirus maintained among avian and mosquito hosts. However, slow mutation rates and the lack of observed pathology suggest that FLAV poses a low medical or veterinary risk [4]. Nonetheless, clinicians should consider FLAV a potential novel pathogen in undiagnosed cases of disease in humans and animals throughout the Americas. Other knowledge gaps for FLAV include transmission ecology, including long-distance dispersal, and interspecific interaction between HPV, FLAV and other microorganisms.

Acknowledgements

Funding was provided from the Centers for Disease Control and Prevention (CDC), the University of Denver's Partners in Scholarship Program and the National Science Foundation Graduate Research Fellowship Program under Grant 1252521. We thank Robert S. Lanciotti for conducting some initial diagnostic testing and providing helpful guidance.

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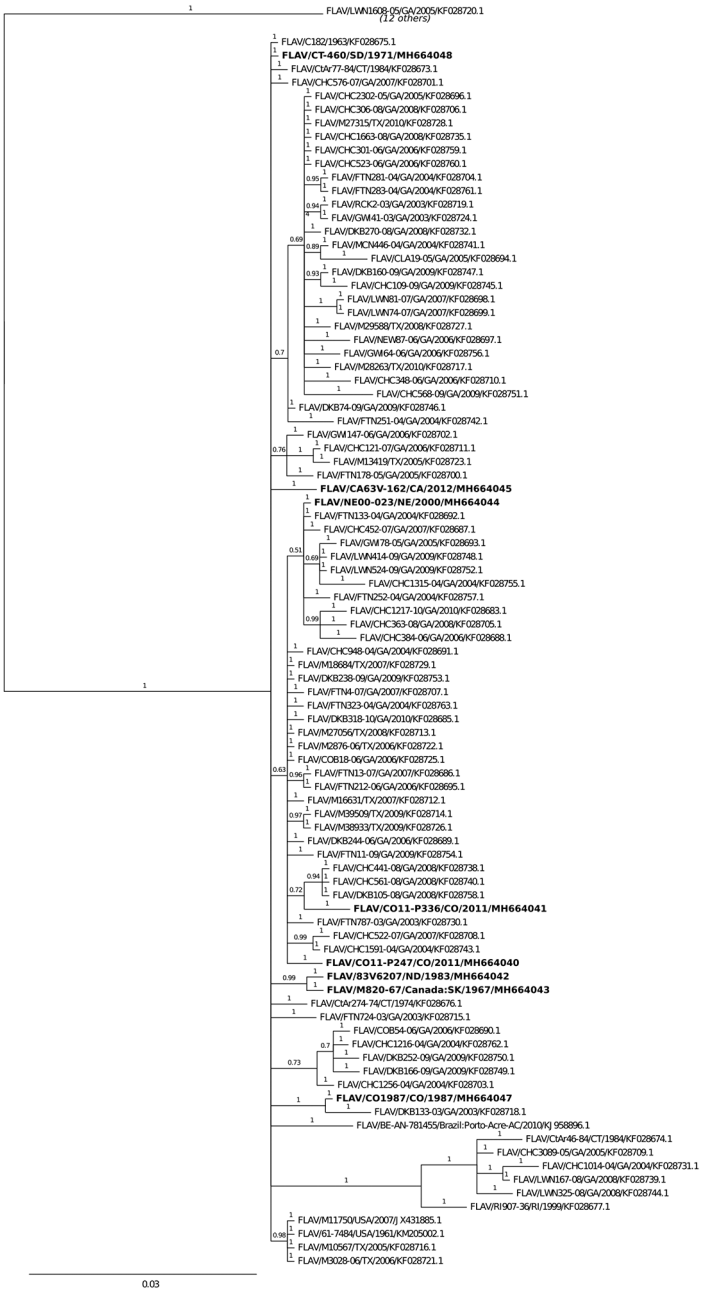

Fig. 1.

A phylogenetic tree inferred using the U1 pseudogene from 117 Flanders hapavirus (FLAV) isolates rooted with Hart Park hapavirus as an outgroup. The alignment was done using MAFFT with the L-INS-i algorithm, and the GTR+I evolutionary model was selected by Jmodeltest2. MrBayes was used for Markov chain Monte Carlo (MCMC) analysis. This figure was produced in FigTree v1.4.2. Posterior probabilities are listed at each node. FLAV isolates from western North America are listed in bold font. The Hart Park virus outgroup is not pictured

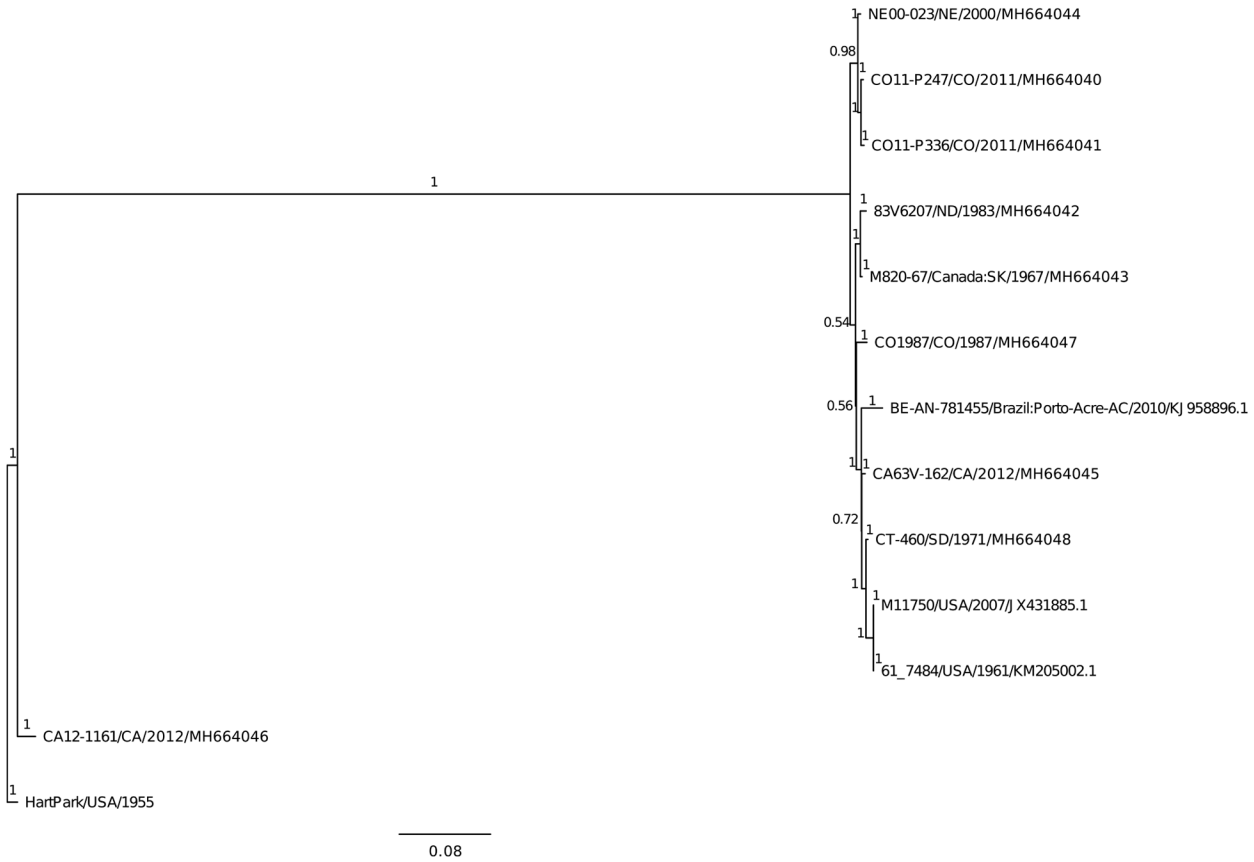

Fig. 2.

Eight nuclear genes (nucleoprotein, RNA polymerase, phosphoprotein, matrix protein, glycoprotein, and pseudoprotein U1-U3) for 15 taxa were used to complete a full-genome phylogenetic analysis of Flanders virus using Hart Park virus as an outgroup. Alignment was done using the MAFFT L-INS-i algorithm. Partition Finder was used to select the best-fit partitioning schemes for the eight genes. MrBayes was used for a Markov chain Monte Carlo (MCMC) analysis. This figure was produced in FigTree v1.4.2. Posterior probabilities are listed at nodes. Isolates in bold font are from western North America

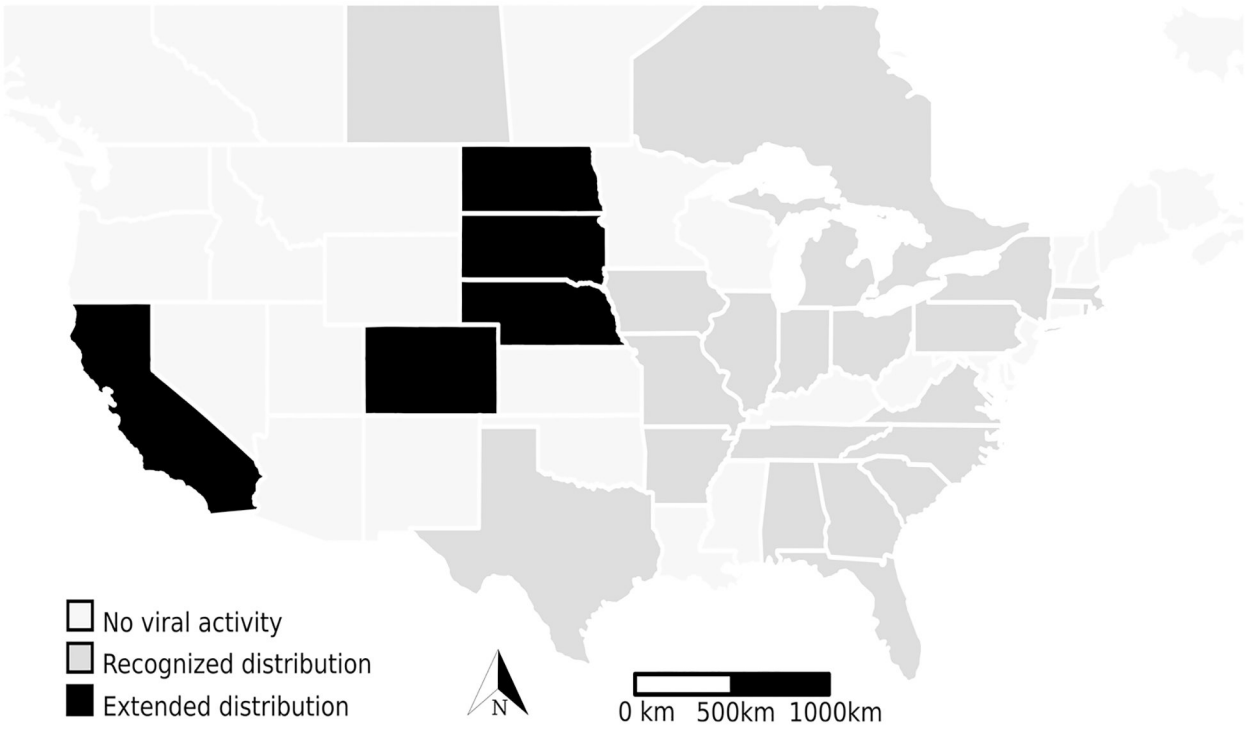

Fig. 3.

The updated state/province-based geographic range of Flanders hapavirus in the United States and Canada is illustrated utilizing published literature, metadata available in the NCBI GenBank database, and results from this study [1, 3, 17–29]. Regions of known circulation prior to this study are indicated in light gray. Novel regions with FLAV activity highlighted by this study are in black. FLAV activity in Mexico and Brazil based on GenBank accession numbers KJ958896 and KF028673 are not pictured

Table 1

Metadata describing western Flanders virus isolates used in this study

Strain name	Origin	Host species	Collection date	Isolation comments
CA12–1161	California	*Culex tarsalis*	29-Jul-12	Not isolated
CO11-P336	Colorado	*Cx. tarsalis*	17-Aug-11	One Vero cell passage
CO11-P247	Colorado	*Cx. tarsalis*	03-Aug-11	One Vero cell passage
NE00–023	Nebraska	*Branta canadensis*	29-Jun-00	One Vero cell passage
CO1987	Colorado	*Cx. tarsalis*	1-Sep-87	One Vero cell passage
83V6207	North Dakota	*Culicidae* spp.	13-Aug-83	One Vero cell passage
CT-460	South Dakota	*Cx. tarsalis*	3-Aug-71	One Vero cell passage
M820–67	Saskatchewan	*Cx. tarsalis*	1967	Unknown passage
CA63V-162	California	*Cx. tarsalis*	1963	One mouse brain passage and one Vero cell passage